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NEW APPROACHES TO ATTENUATED HEPATITIS A VACCINE DEVELOPMENT:

CLONING AND SEQUENCING OF CELL-CULTURE ADAPTED VIRAL cDNA

ANNUAL REPORT

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April 1, 1989

Supported by

U.S. ARMY MEDICAL RESEARCH AND DEVELOPMENT COMMAND
Fort Detrick, Frederick Maryland 21701

Contract No. DAMD17-85-C-5272

University of North Carolina at Chapel Hill
Chapel Hill, North Carolina 27599-7030

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90 03 08 078

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1a. REPORT SECURITY CLASSIFICATION	1b. RESTRICTIVE MARKINGS
Unclassified	

2a. SECURITY CLASSIFICATION AUTHORITY	3. DISTRIBUTION/AVAILABILITY OF REPORT
	Approved for public release;
2b. DECLASSIFICATION/DOWNGRADING SCHEDULE	distribution unlimited

4. PERFORMING ORGANIZATION REPORT NUMBER(S)

5. MONITORING ORGANIZATION REPORT NUMBER(S)

6a. NAME OF PERFORMING ORGANIZATION	6b. OFFICE SYMBOL (If applicable)	7a. NAME OF MONITORING ORGANIZATION
University of North Carolina		

6c. ADDRESS (City, State, and ZIP Code)	7b. ADDRESS (City, State, and ZIP Code)
Chapel Hill, North Carolina 27599-7030	

8a. NAME OF FUNDING/SPONSORING ORGANIZATION U.S. Army Medical Research & Development Command	8b. OFFICE SYMBOL (If applicable)	9. PROCUREMENT INSTRUMENT IDENTIFICATION NUMBER
		Contract No. DAMD17-85-C-5272

8c. ADDRESS (City, State, and ZIP Code)	10. SOURCE OF FUNDING NUMBERS			
	PROGRAM ELEMENT NO.	PROJECT NO. 3M1-	TASK NO.	WORK UNIT ACCESSION NO.
Fort Detrick Frederick, Maryland 21701-5012	61102A	61102BS10	AA	055

11. TITLE (Include Security Classification)

New Approaches to Attenuated Hepatitis A Vaccine Development: Cloning and Sequencing of Cell-Culture Adapted Viral cDNA

12. PERSONAL AUTHOR(S)

Stanley M. Lemon, M.D.

13a. TYPE OF REPORT	13b. TIME COVERED	14. DATE OF REPORT (Year, Month, Day)	15. PAGE COUNT
Annual Report	FROM 9/15/87 3/14/89	1989 April 1	36

16. SUPPLEMENTARY NOTATION

17. COSATI CODES			18. SUBJECT TERMS (Continue on reverse if necessary and identify by block number)
FIELD	GROUP	SUB-GROUP	Hepatitis A Vaccine, Molecular Cloning and Hybridization,
06	13		Strain Differences, Attenuation, RA I
06	03		

19. ABSTRACT (Continue on reverse if necessary and identify by block number)

An infectious cDNA clone derived from virulent HAV would be of considerable value in experiments designed to determine the molecular basis of attenuation, and would provide the basis for new approaches to developing candidate attenuated viruses. In an effort to assemble such a clone, partial inserts from ten HM175 cDNA clones were assembled into a single construct containing the consensus p16 HM175 sequence. Flanking homopolymeric dC-dG tails derived from the original cloning procedure were removed, and the full length sequence of p16 HAV cDNA was inserted between the HindIII and XbaI sites of the transcription vector pGEM3. At the conclusion of the contract period, the infectivity of this construct (pHAV/p16) was under evaluation. The sequence of the P1 genomic regions of two plaque-purified, cytopathic variants of HM175 virus was determined from virion RNA; one of these variants was shown to be a spontaneous neutralization escape mutant. A novel immunoaffinity-linked nucleic acid amplification

20. DISTRIBUTION/AVAILABILITY OF ABSTRACT	21. ABSTRACT SECURITY CLASSIFICATION
☐ UNCLASSIFIED/UNLIMITED ☒ SAME AS RPT. ☐ DTIC USERS	Unclassified

22a. NAME OF RESPONSIBLE INDIVIDUAL	22b. TELEPHONE (Include Area Code)	22c. OFFICE SYMBOL
Virginia M. Miller	301/663-7325	SGRD-RMI-S

DD Form 1473, JUN 86 — Previous editions are obsolete.

19. Abstract continued...

system (antigen-capture/polymerase chain reaction, or AC/PCR) capable of the strain-specific detection of HAV in clinical specimens was developed and evaluated. Molecular cloning and partial sequencing of the genome of PA21 strain HAV was undertaken in an effort to determine the extent of genetic divergence from human HAV.

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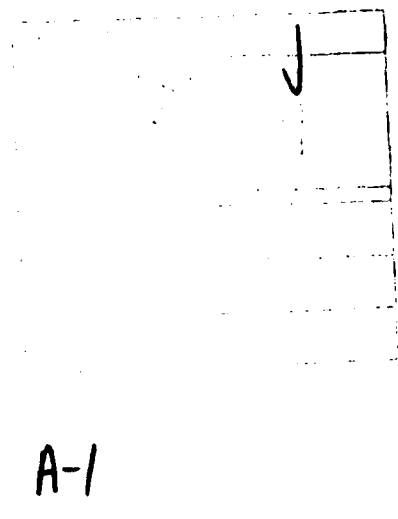
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SUMMARY

During the first two years of this contract, we molecularly cloned and sequenced the genome of the cell culture-adapted but still virulent p16 variant of HM175 strain hepatitis A virus (HAV). The identification of mutations present within this genome have provided useful information concerning the molecular basis of adaptation of the virus to growth in cell culture. Because an infectious clone derived from virulent HAV would be of considerable value in determining the molecular basis of attenuation, cDNA from ten HAV cDNA clones was assembled into a single construct containing the consensus p16 HM175 sequence flanking dC-dG tails derived from the original homopolymeric cloning procedure were removed, and the full length sequence of p16 HAV cDNA was inserted between the HindIII and XbaI sites of the plasmid vector pGEM3. At the conclusion of the contract period, the infectivity of this construct (pHAV/p16) was under evaluation.

The pHM175 variant of HAV, recovered by disruption of persistently infected continuous green monkey kidney (BS-C-1) cells, induces a cytopathic effect during serial passage in either BS-C-1 or fetal rhesus kidney (FRhK-4) cells. Virus neutralization and epitope-specific radioimmunofocus assays showed that pHM175 virus stock contained two virion populations, one with altered antigenicity including neutralization-resistance to monoclonal antibody K24F2, and the other with normal antigenic characteristics. Replication of the antigenic variant (K24F2$^-$) was favored over replication of virus with normal antigenic phenotype (K24F2$^+$) during persistent infection in BS-C-1 cells, while K24F2$^+$ virus replicated to higher titers than K24F2$^-$ virus during subsequent serial passage in FRhK-4 cells. Both virus types were cytopathic in monkey kidney cell cultures, and both displayed a rapid replication phenotype when compared with the parental virus used to establish the original persistent infection (p16 HM175 virus). The sequence of the P1 genomic regions of plaque-purified variants of each virus type was determined from virion RNA. Compared with p16 HM175 virus, amino acid replacements were present in K24F2$^-$ virus (43c clone) at   residue 70 of VP3 and residues 197 and 276 of VP1; changes in K24F2$^+$ virus (181   e) were at residues 91 of VP3 and 271 of VP1. These mutations did not reduce the thermal stability of the virion. Although cytopathic viruses do not share a unique capsid mutation, the emergence of an antigenic variant during persistent infection suggests that changes in capsid proteins may influence virus replication in cell culture.

We developed a novel immunoaffinity-linked nucleic acid amplification system (antigen-capture/polymerase chain reaction, or AC/PCR) for detection of HAV in clinical specimens. We have applied this new procedure to the study of the molecular epidemiology of HAV. Immunoaffinity capture of virus, synthesis of viral cDNA, and amplification of cDNA by a polymerase chain reaction (PCR) were carried out sequentially in a single tube. This approach simplified sample preparation and significantly enhanced the specificity of conventional PCR. AC/PCR consistently detected less than one cell culture infectious unit of virus in 80 ul of sample. Sequencing of AC/PCR reaction products from 13 HAV strains demonstrated remarkable conservation at the nucleotide level, but suggested the presence of distinct virus genotypes circulating within

geographically defined regions. Virus strains recovered from two military epidemics of hepatitis A were identical in sequence, providing evidence for a previously unrecognized epidemiologic link between these outbreaks.

PA21, a strain of HAV recovered from a naturally infected captive owl monkey, is indistinguishable from human HAV in polyclonal radioimmunoassays and cross-neutralization studies. However, cDNA-RNA hybridization has suggested a significant difference at the genomic level between PA21 and a reference human virus, HM175. Molecular cloning and partial sequencing of the genome of PA21 was undertaken in an effort to determine the extent of genetic divergence from human HAV. The nucleotide sequence of the P1 region of the PA21 genome had only 83.2% identity with HM175 virus, a difference approximately twice as great as that found between any two previously studied human strains. Most nucleotide changes were in third base positions, and the amino acid sequence of the capsid proteins was largely conserved. Amino acid replacements were clustered in the carboxy terminus of VP1 and the amino terminal regions of VP2 and VP1. Similar genetic divergence was evident in P2 and P3 genomic regions, but the 5' and 3' noncoding regions were relatively conserved (89.2% and 93.7% nucleotide identity respectively). These data indicate that PA21 virus represents a unique genotype of HAV and suggest the existence of an ecologically isolated niche for HAV among feral owl monkeys.

FOREWORD

Citations of commercial organizations and trade names in this report do not constitute an official Department of the Army endorsement or approval of the products or services of these organizations.

The investigators have abided by the National Institutes of Health Guidelines for Research Involving Recombinant DNA Molecules (April 1982) and the Administrative Practices Supplement.

TABLE OF CONTENTS

EXPERIMENTAL APPROACH

1. Adaptation and passage of hepatitis A virus (HAV) in cell culture regularly results in reduction in virulence of the virus in primate challenge experiments. During the first two years of this contract, we molecularly cloned and completely sequenced the genome of a cell culture-adapted variant of HAV (16th in vitro passage, or p16 HM175 virus), resulting in the identification of mutations associated with adaptation of this strain of HAV to growth in cell culture (Jansen et al., 1988). We also demonstrated that HM175 virus derived from p16 virus remained virulent in owl monkeys at the 22nd in vitro passage level (Lemon et al., 1989). Thus the p16 HM175 cDNA clones were found to represent the sequence of a cell culture-adapted yet virulent virus, and thereby offered the potential for construction of a genomic-length, infectious (virulent) cDNA clone. Such a construct would be invaluable in determining the attenuation determinants of HAV (as the only existing infectious HAV clone is derived from a highly attenuated virus variant) (Cohen et al., 1987c), and would permit the construction of new viruses with deletion mutations engineered to reduce virulence. During this contract period, using a small number of subgenomic cDNA clones derived from p16 HM175 (Jansen et al., 1987), we completed construction of a plasmid containing a full-length cDNA copy of the p16 HM175 HAV genome. At the conclusion of this contract period, this clone was being evaluated to determine whether or not it was infectious following transfection as DNA or RNA into permissive cells.

2. We characterized a cytopathic variant of HM175 virus (pHM175 virus, Cromeans, et al., 1987) that was found to be exceptionally well adapted to growth in cell culture. This virus, obtained by passage of persistently infected BS-C-1 cells was determined to be comprised of a mixture of a mutant with a monoclonal antibody neutralization escape phenotype, and virus with normal antigenicity. Each virus type was clonally isolated from agarose overlays taken from radioimmunofocus assay cultures, and sequenced from RNA through the entire P1 region. A specific mutation associated with the neutralization escape phenotype was identified at residue 70 of capsid protein VP3 (at the site of mutations in other neutralization escape variants selected under antibody pressure, Ping et al., 1988). Both the mutant (43c virus) and antigenically normal virus (18f) were found to be cytopathic in BS-C-1 and FRhK-4 cells. Despite capsid mutations in both viruses, and the suggestion in the literature that cytopathic variants of HAV might have reduced thermostability, the stability of both viruses was unchanged during short exposures at elevated temperatures. The growth properties of the 18f variant make it an ideal virus reagent for neutralization tests and production of viral antigen in vitro.

3. The strain-specific identification of HAV in human fecal samples was a major aim of the original contract application, as clinical trials of live and inactivated HAV vaccines will require the availability of a sensitive and specific detection method capable of distinguishing wild-type HAV strains from vaccine virus. This was accomplished by developing an integrated test method incorporating solid-phase antigen-capture, in monoclonal antibody-coated microcentrifuge tubes, heat denaturation of immobilized virus, followed by

6

polymerase chain reaction amplification of reverse-transcribed cDNA and primer-extension sequencing of amplified transcripts. This method was applied to a panel of known virus-positive samples, including both tissue culture-derived materials and human and primate fecal specimens.

4. We molecularly cloned and partially sequenced the genome of PA21 strain HAV, a virus recovered from infected owl monkeys by investigators at WRAIR. Previous hybridization studies had suggested a marked sequence divergence between this virus and other strains of HAV recovered from humans, despite close antigenic relatedness (Lemon et al., 1987). Results of these studies suggest that the PA21 virus represents a unique genotype of HAV and may possibly be an indigenous virus of the owl monkey.

RESEARCH PROGRESS

I. Construction of a genomic-length clone of p16 HM175 HAV cDNA

In an attempt to construct an infectious cDNA clone of virulent HAV, cDNA inserts from p16 clones (Jansen et al., 1988) have been used to assemble a genomic-length cDNA construct. This was accomplished by cleavage and ligation of overlapping inserts at convenient restriction enzyme sites (Figure 1). Wild-type HM175 cDNA (Ticehurst et al., 1983; Cohen et al., 1987a) containing sequence identical to the p16 consensus sequence was used in areas of the genome where no convenient enzyme site was available in the p16 cDNA clones, or the sequence of individual p16 clones contained unique mutations not present in other clones derived from that region of the genome. Such mutations were not considered to be representative of the consensus p16 HM175 sequence. All ligation sites were subsequently sequenced to preclude the introduction of any sequence errors during the cloning procedure. The resulting full-length construct (pHAV$_{ch}$p16), cloned into the PstI site of pBR322, contained flanking dC-dG tails derived from the original cDNA-RNA cloning procedure (Jansen et al., 1988). This construct was transfected into BS-C-1 cells as supercoiled plasmid DNA, with calcium phosphate facilitation. The transfection control was pHAV/7 cDNA, derived from attenuated p35 HM175 virus (Cohen et al., 1987c) and obtained from R. Purcell of the NIAID. Transfected cell monolayers were grown under agarose overlays for two weeks at 36°C. The monolayers were then acetone fixed and stained with radiolabelled anti-HAV IgG and foci of virus replication detected by autoradiography (radioimmunofocus assay). HAV replication was detected with pHAV/7, but not pHAV$_{ch}$p16.

Cohen et al. (1987c) have suggested that homopolymeric dC-dG tails render genomic-length cDNA constructs of HAV non-infectious. We therefore removed the dC-dG tail at the 5' end of the construct using a polymerase chain reaction (PCR) based approach. Nucleotides 0-885 of the p16 genome were amplified from clone pHAV$_{ch}$119 (see Report Number 2) using a genomic-sense PCR primer from nucleotide 870-885 and an antigenomic-sense primer containing HAV specific sequence from nucleotides 0-14 with an additional seven nucleotide HindIII restriction enzyme site placed immediately 5' of nucleotide 0 (note: nucleotide 0 is a terminally redundant U in the p16 genome, Jansen et al.,

7

1988). The amplified product was subsequently cleaved with HindIII (nt 0) and
XbaI (nt 745) and cloned into the pGEM3 transcription vector (Promega) between
the HindIII and XbaI sites of the multiple cloning region, to create pHAV5'.
A full-length construct of p16 lacking dC-dG tails at both the 5' and 3' ends
was then assembled by ligation of fragments from pHAV5' (HAV nt 0-24),
pHAV$_{ch}$p16 (nt 25-7003), and pHAV/7 (nt 7004-7505) (Figure 2). The resulting
construct (pHAV/p16) contains the consensus p16 HM175 sequence, minus a
mutation at 7430 of the 3' noncoding region (Jansen et al., 1988), inserted
between the HindIII and XbaI sites of the multiple cloning region of pGEM3 and
without flanking dC-dG homopolymeric tails. pHAV/7 cDNA (Cohen et al., 1987c)
provided a convenient 3' end for the construct (nt 7004-7505) because it
lacked a dC-dG tail and has a poly-A tail of sufficient length for
infectivity. cDNA in this region was originally derived from wild-type HM175
(Ticehurst et al., 1983) and differs from the p16 sequence at only one base
(nt 7430 of the 3' noncoding region) (see Report Number 2). Absence of this
nucleotide change has no demonstrable effect on HAV cDNA infectivity (Cohen et
al., 1987c).

At the conclusion of the contract period, the infectivity of the
pHAV/p16 construct was under evaluation.

II. Characterization and partial nucleotide sequencing of the cytopathic variants selected from pHM175 HAV

Rationale HAV is unique among human picornaviruses with respect to its
tropism for liver cells and its capacity to induce acute hepatocellular injury
(Lemon, 1985). Although the events accompanying HAV replication in vivo
remain poorly defined, replication of the virus in vitro has been well
characterized. A variety of primate cell types have been shown to be
permissive for HAV, but most wild-type virus isolates replicate very slowly in
cell culture (Binn et al., 1984; Provost and Hilleman, 1979; Flehmig, 1980;
Daemer et al., 1981). Final yields of virus are relatively low compared with
yields achieved with virus that has been adapted to growth in cell culture
through successive passage in vitro. However, even highly cell culture-
adapted virus replicates considerably more slowly and less efficiently than
poliovirus. In almost all cases, wild-type or low passage virus does not
induce visible cytopathic effects, and there is no evidence that HAV
interferes with host cell macromolecular synthesis (Gauss-Müller and
Deinhardt, 1984). In vitro infections generally result in the establishment
of persistent infection (Vallbracht et al., 1984), a curious finding since
viral persistence is not known to occur in vivo (Lemon, 1985).

In parallel with its adaptation to growth in cell culture, the ability
of more highly passaged virus to replicate in vivo appears to become
progressively restricted. Low titer or absent antibody responses in
susceptible primates that have been challenged with highly cell culture
passaged virus suggest that replication of such virus is limited or
nonexistent in vivo (Provost et al., 1982). Less highly passaged virus
retains the ability to replicate in vivo, but may be relatively attenuated in
its ability to cause hepatocellular disease (Karron et al., 1988; Provost et
al., 1982). The molecular basis of this change in host range accompanying

adaptation of the virus to cell culture is thus of interest with respect to vaccine development. Mutations associated with adaptation of HAV to growth in vitro have been identified in two independent isolates of the HM175 strain of HAV, and have been shown to involve nearly all regions of the genome (Cohen et al., 1987b; Jansen et al., 1988). However, it is not known which of these mutations are of greatest importance in adaptation of HAV to growth in vitro, nor is the nature of the general restriction to growth of wild-type HAV in cell culture understood.

Recently, several unique variants of HAV have been reported to induce a cytopathic effect in monkey kidney cells in vitro (Venuti et al., 1985; Anderson, 1987; Cromeans et al., 1987; Nasser and Metcalf, 1987). In at least three instances, cytopathic variants have emerged during continued passage of persistently infected cell cultures (Anderson, 1987; Cromeans et al., 1987; Nasser and Metcalf, 1987). An important attribute of these cytopathic variants, each derived from the HM175 strain of HAV, is that they appear to be uniquely well adapted to growth in cell culture. One such variant (Cromeans et al., 1987) replicates significantly more rapidly than its noncytopathic precursor, with maximum viral yields reached as early as 40 hours after inoculation of cells under one-step growth conditions (Cromeans et al., 1989). In further characterizing this cytopathic variant, we noted that the virus had spontaneously acquired a mutation within an immunodominant neutralization epitope (Ping et al., 1988). This finding prompted a detailed study of changes in capsid proteins accumulating during persistent HAV infection in vitro, and their effects on antigenicity and temperature stability of the virus.

Cytopathic HM175 Virus   The HM175 strain HAV was adapted to growth in primary African green monkey kidney (AGMK) cells as described previously (Binn et al., 1984). Several variants derived from this virus strain have been studied in detail. "p16 HM175" virus had undergone a total of 16 cell culture passages: 6 passages in BS-C-1 cells following 10 passages in AGMK cells (see Report Number 2). Persistent infection of BS-C-1 cells with p16 HM175 virus was established as described previously (Cromeans et al., 1987). "pHM175" virus was recovered by disruption of these persistently infected cells after 21 to 23 subculture passages made at 2 to 4 week intervals. Unless otherwise noted, pHM175 virus stock had been serially passaged 4 times at low m.o.i. in FRhK-4 cells. By "serial passage", we mean the passage of virus recovered by disruption of infected cells. "43c" virus was twice plaque-purified from neutral red-stained overlays of FRhK-4 cells inoculated with pHM175 virus, and was selected for its ability to induce clearly visible plaques in this cell line. 43c stock virus was prepared in FRhK-4 cells. "18f" virus was twice clonally isolated from agarose overlying foci of viral replication in radioimmunofocus assays (Lemon and Jansen, 1985) (see below) of BS-C-1 cells inoculated with pHM175 virus; it was selected for continued reactivity with monoclonal antibody K24F2. 18f stock virus was prepared in BS-C-1 cells.

Antigenic variation among cytopathic HM175 strain HAV variants   In previous studies, persistent HAV infection was established by inoculation of BS-C-1 cells with p16 HM175 virus (Cromeans et al., 1987). These cells were subsequently subcultured at 2-4 week intervals. Rapidly replicating virus

(pHM175) was recovered by disruption of the persistently infected cells after 21-23 subculture passages, and was shown to induce a cytopathic effect in both BS-C-1 cells and high-passage level FRhK-4 cells (Cromeans et al., 1987; Cromeans et al., 1989). We confirmed these results by establishing a plaque assay with HAV recovered by disruption of persistently infected BS-C-1 cells at the 21st-23rd passage level. Conventional plaque assays were carried out with either FRhK-4 or BS-C-1 cells. Following inoculation with pHM175 virus, cell sheets were overlaid with 0.5% agarose (Seakem ME, Rockland, ME) containing 2.0% heat-inactivated fetal calf serum and placed at 35 °C in a humidified environment with 5% CO_2. Six days later, a second overlay containing 0.5% agarose, 25mM $MgCl_2$ and neutral red (final concentration 0.02%) was added. Visible plaques were counted 6-24 hours later.

A clonal isolate (43c virus) was twice plaque-purified from the pHM175 virus harvest. When cell monolayers inoculated with 43c virus and overlaid with agarose were sequentially stained with neutral red and then processed for detection of radioimmunofoci, there was excellent concordance between the numbers of visible plaques and radioimmunofoci (not shown). Only occasional foci of viral replication that were visualized by staining with [^{125}-I]-labelled polyclonal anti-HAV in the radioimmunofocus assay were not recognized as plaques following the staining of cell sheets with neutral red. Furthermore, autoradiograms of the [^{125}I]-anti-HAV stained cell sheets often showed replication foci with central areas of cell necrosis, confirming the HAV-specific nature of the cytopathic effect.

As further proof that HAV was responsible for plaque formation, neutralization of the plaque-purified 43c virus was attempted with murine monoclonal antibodies to HAV in a plaque-reduction neutralization test. Surprisingly, these tests revealed that 43c virus was susceptible to neutralization with antibody K34C8 (83% plaque reduction at 0.1 mg/ml antibody), but resistant to antibody K24F2 (only 4.4% plaque reduction). These results were subsequently confirmed in a radioimmunofocus inhibition assay. The K34C8 and K24F2 monoclonal antibodies were raised against the HM790 strain of HAV (MacGregor et al., 1983), and both have been shown previously to neutralize HM175 virus following its adaptation to growth in cell culture (Stapleton and Lemon, 1987). These results thus indicated that passage of HM175 virus in persistently infected BS-C-1 cells and subsequent plaque selection of virus capable of inducing a cytopathic effect, all in the absence of applied antibody pressure, had resulted in isolation of virus with altered antigenicity and a K24F2 neutralization escape phenotype.

Relative replication advantages of K24F2-resistant and K24F2-sensitive virus during acute and persistent infections Because an antigenic variant of HM175 virus had been selected during persistent infection in vitro, we utilized an epitope-specific radioimmunofocus assay to determine the titer and proportion of antigenically variant virus present in virus harvests from the persistently infected cells. We examined several different passage levels of the persistently infected BS-C-1 cells, and harvests made from subsequent serial passage of pHM175 virus in BS-C-1 and FRhK-4 cells. The epitope-specific radioimmunofocus assay involved sequential staining of infected cell sheets with radioiodinated monoclonal (K24F2) and polyclonal (JC) antibodies.

Replication foci of virus bearing the K24F2 epitope (K24F2$^+$, wild-type antigenic phenotype) were visualized by autoradiography following reaction with either the monoclonal antibody or the polyclonal antibody, while foci of K24F2 resistant mutants (K24F2$^-$ phenotype) were recognized only by staining with polyclonal antibody. This approach revealed that 99% of the infectious virus particles present in disrupted cell lysates from the 23rd passage of persistently infected BS-C-1 cells were of the mutant K24F2$^-$ phenotype. However, with subsequent serial passage of this virus in FRhK-4 cells, virus with a normal antigenic phenotype (K24F2$^+$) was selected over K24F2$^-$ virus. The K24F2$^+$ virus represented 92-97% of all infectious virus after 3-4 serial passages in FRhK-4 cells.

The results of these and other assays are shown in greater detail in Figure 3. During the last three subculture passages of persistently infected BS-C-1 cells, the majority of virus present (>94%) was K24F2$^-$ (Figure 3A). This virus was rapidly replaced with K24F2$^+$ virus during subsequent serial 3 day passages in FRhK-4 cells (Figure 3B). Both mutant and nonmutant phenotypes replicated to higher titers upon recovery from persistently infected BS-C-1 cells and subsequent serial passage in FRhK-4 cells. However, the nonmutant K24F2$^+$ virus replicated to significantly greater titers and became the predominant virus in a mixture of virus types following the second serial passage in FRhK-4 cells.

To determine whether the overgrowth of the K24F2$^+$ wild-type antigenic phenotype shown in Figure 3B reflected a change in host cell type (BS-C-1 cells to FRhK-4 cells) or a change in passage conditions (persistently infected cell passage to serial passage), virus harvested from the 23rd passage level of the persistently-infected cells was serially passaged in BS-C-1 cells at 7 day intervals (Figure 3D). As happened during the shift to serial passage in FRhK-4 cells, the yield of both virus types increased with serial passage in BS-C-1 cells. However, K24F2$^+$ virus demonstrated no selective advantage over K24F2$^-$ virus under these growth conditions (Figure 3D), suggesting that the selection of K24F2$^+$ virus which occurred in FRhK-4 cells (Figure 3B) may reflect a host cell-specific replication advantage of K24F2$^+$ virus.

To determine whether K24F2$^-$ virus would be reselected if persistent infection was reestablished with the predominantly K24F2$^+$ virus recovered from FRhK-4 cells after serial passage, BS-C-1 cells were inoculated with virus harvested after the 4th serial passage in FRhK-4 cells (92% K24F2$^+$, Figure 3B) and these cells were subcultured at weekly intervals for 4 weeks. Cell lysates were monitored for the proportion of K24F2$^-$ and K24F2$^+$ phenotypes (Figure 3C). The yield of each virus type dropped significantly in harvests made after the third passage as persistently infected cells. The proportion of mutant K24F2$^-$ virus increased with each passage, however, and after four passages as persistently infected cells, K24F2$^-$ virus represented 19% of the total virus yield. Further passage of these persistently infected cells was not done. The data shown in Figure 3A and 3C thus suggest that the K24F2$^-$ mutant may have a significant replication advantage over K24F2$^+$ virus during passage of persistently infected BS-C-1 cells.

Both K24F2-resistant and K24F2-sensitive pHM175 virus induce a
cytopathic effect Three clonally isolated viruses (43c, 12d, and 12e) were
picked from plaques forming in FRhK-4 cells inoculated with the cytopathic
pHM175 virus; each of these plaque-purified viruses was found to have a K24F2⁻
phenotype. However, since the data shown in Figure 3B suggested that K24F2⁺
virus in pHM175 virus harvests shared a rapidly replicating phenotype with
K24F2⁻ virus, it was of interest to determine whether K24F2⁺ virus was also
capable of inducing a cytopathic effect. Accordingly, a K24F2⁺ virus variant
(18f virus) was clonally isolated from the agarose overlay of an epitope-
specific radioimmunofocus assay carried out in BS-C-1 cells. Unlike the 43c
virus which was selected for its ability to induce distinct plaques, 18f virus
was selected for its continuing ability to react with K24F2 antibody. 18f
virus was shown to have a rapidly replicating phenotype, as it formed large
radioimmunofoci within 7 days after inoculation of cells. Similar size
replication foci require approximately 14 days incubation with the parental
p16 virus. Furthermore, 18f virus produced cytopathic effects in both BS-C-1
and FRhK-4 cells matching those found with the K24F2⁻ 43c virus. These
cytopathic changes were similar to those described previously (Anderson, 1987;
Cromeans et al., 1987), and included the rounding-up of cells followed by
their release from the cell sheet.

To compare the cytopathic effect induced by the K24F2⁺ 18f and the
K24F2⁻ 43c viruses, parallel cultures of FRhK-4 cells were inoculated with 18f
and 43c viruses at low m.o.i. (approximately 0.01). Additional FRhK-4 cells
were inoculated with the parental p16 HM175 virus which had never been
subjected to persistent passage. These cultures were monitored for cytopathic
effect, release of viral antigen into supernatant fluids, and development of
intracellular viral antigen (Table 1). This experiment demonstrated an
earlier and somewhat greater cytopathic effect with 43c virus than with 18f
virus. Cytopathic changes were not seen with the p16 HM175 virus, even when
infected cells were held for 2-3 weeks. Release of viral antigen into cell
culture supernatant fluids was noted with 18f but not 43c virus, and
intracellular antigen accumulation was greater with 18f than with 43c (Table
1). However, the detection of antigen may have been biased by the altered
antigenicity of 43c, even in polyclonal radioimmunoassays (see below). 18f
virus generated distinct plaques in BS-C-1 cells maintained under agarose and
stained with neutral red (not shown). These studies thus demonstrated that
escape from neutralization by K24F2 was neither necessary for nor specifically
associated with the ability of HM175 virus variants to generate a cytopathic
effect in cell culture.

Capsid mutations associated with K24F2 neutralization escape, persistent
infection, and cytopathic phenotype To determine capsid protein mutations in
43c and 18f viruses, we sequenced the P1 regions of genomic RNA extracted from
these variants and compared these sequences with those of wild-type (3
marmoset passages) (Cohen et al., 1987a) and p16 HM175 virus (Jansen et al.,
1988) (Table 2). p16 HM175 virus differs from wild-type HAV not adapted to
growth in cell culture by a total of 19 mutations throughout its genome (see
Report Number 2, Jansen et al., 1988). Only two of these mutations (one
silent) are in the capsid-encoding P1 region. The single nonsilent mutation
predicts a change from lysine to arginine at residue 54 of VP2 (by convention,

12

residue 2-054). The change at residue 2-054 which was found in the parent p16 virus was also present in both 43c and 18f viruses, as was the single silent mutation at base 1742. However, in the K24F2$^+$ 18f virus, additional mutations were identified by RNA sequencing at residues 3-091 (threonine to lysine), and 1-271 (serine to proline) (Table 2). Interestingly, the substitution at residue 3-091 in 18f virus occurred as a result of a mutation in the base (1741) adjoining the silent mutation found in all the cell culture-adapted variants, resulting in a change in two of the bases present in the wild-type codon. As previously reported (Ping et al., 1988), mutations were found at residues 3-070 (aspartic acid to alanine), 1-197 (asparagine to serine) and 1-276 (methionine to valine) of the K24F2$^-$ 43c virus. Thus, remarkably similar mutations were found in the 43c and 18f cytopathic viruses, with mutations present in both viruses in regions bounded by residues 70 to 91 of VP3, and 271 to 276 of VP1. Outside these regions, only two additional mutations (from the noncytopathic p16 virus) were noted in the P1 genomic sequences of 43c and 18f viruses, one of which was silent (base 2684 of 18f). These data thus indicate a relatively low rate of mutation in the P1 region of the genome during persistent infection over the course of almost one year.

Comparative antigenicity of cytopathic 43c and 18f HM175 variants

Because capsid mutations were identified in both 43c and 18f viruses, we carried out a detailed comparison of the antigenicity of these viruses and the noncytopathic p16 parent virus, using a panel of neutralizing murine monoclonal antibodies assembled from several laboratories. Standardized quantities of each virus (determined by cDNA-RNA hybridization) were tested for ability to bind monoclonal antibodies in an indirect radioimmunoassay (Ping et al., 1988). While these assays demonstrated that 43c virus was poorly recognized by many of the monoclonal antibodies, the 18f variant was indistinguishable from the noncytopathic p16 virus (Figure 4). As further evidence of the antigenic differences between these viruses, standardized quantities of each virus were tested in a polyclonal radioimmunoassay. These results indicated that the few capsid mutations present in 43c virus resulted in a marked reduction in the antigenicity of the virus that was detectable in a polyclonal antibody-based assay. This finding supports the concept that the HAV capsid displays an immunodominant antigenic site on its surface (Stapleton and Lemon, 1987; Ping et al., 1988). In contrast, the mutations present in the 18f capsid did not result in demonstrable changes in antigenicity in either monoclonal or polyclonal radioimmunoassays. These findings have been confirmed in subsequent monoclonal antibody neutralization assays.

Thermal stability of cytopathic HM175 variants

Since it has been suggested that another cytopathic variant arising from HM175 virus during persistent infection in vitro might have reduced thermal stability (Anderson, 1987), we determined whether the mutations present in 43c and 18f variants affected the thermal stability of these viruses. Crude cell lysates containing p16, 43c and 18f viruses were heated for 10 min at temperatures ranging from 40 °C to 90 °C, then assayed for infectious virus at 35 °C (Figure 5), and for HAV antigen detectable in a polyclonal solid-phase radioimmunoassay. Incubations were carried out in the absence (Figure 5A) or presence (Figure 5B) of additional Mg^{++} (1 M MgCl$_2$), as this divalent cation is known to stabilize the HAV capsid against heat denaturation (Siegl et al.,

1984). These studies failed to demonstrate reproducible differences between the thermal stability of the p16 virus and that of the cytopathic 43c and 18f variants, indicating that the rapidly replicating cytopathic phenotype was not associated with reduced thermal stability of the virus capsid With increasing temperatures, significant decrements in infectivity of each virus were noted before comparable reductions in antigenicity, suggesting heat-related conversion of virus to noninfectious particles that retained antigenicity. As expected, the addition of 1 M Mg^{++} resulted in an increase of approximately 20 °C in the 50% survival point of all three viruses.

III. Development of an antigen-capture/polymerase chain reaction method for strain-specific identification of HAV in human fecal materials

Rationale Current understanding of the molecular epidemiology of HAV has been limited by the absence of straightforward methods for isolating virus and distinguishing between virus strains. Certain differentiation between HAV strains has been accomplished only by "RNA fingerprinting", or by a comparison of nucleotide sequences derived from molecularly cloned viral cDNA (Weitz and Siegl, 1985; Ticehurst et al., 1989). Such approaches require considerable quantities of virus or the adaptation of virus to growth in cell culture, in itself a difficult task and one which may lead to mutations within genomic RNA (see Report Number 2). These labor intensive methods of distinguishing between viral strains are not directly applicable to the small quantities of virus usually present in clinical specimens, and have thus been of limited use in epidemiologic studies. To overcome these limitations and develop a molecular approach to studying the epidemiology of HAV, we turned to the polymerase chain reaction (PCR) as a means of amplifying viral nucleic acid present in clinical specimens.

PCR involves the selective, enzymatic amplification of a segment of a DNA molecule and may effect a greater than 10^6-fold enrichment of a target DNA sequence (Saiki et al., 1985, 1988). The exquisite sensitivity of this procedure makes it an attractive approach to viral diagnosis, but also leads to potential problems with respect to specificity. Contamination of clinical specimens with miniscule quantities of recombinant nucleic acid present in the laboratory environment may lead to false-positive PCR results, and in some cases random priming of complex mixtures of non-specific nucleic acids may lead to amplification of stochastic reaction products. Moreover, typical methods for preparing viral nucleic acid for PCR are tedious, involve multiple manipulations, and are a potential source of contamination. To reduce these potential problems, we developed an integrated immunoaffinity/PCR procedure (antigen capture/polymerase chain reaction assay, or AC/PCR) for selective amplification of antigen-associated viral nucleic acid. Virus present in crude fecal suspensions was captured by specific murine monoclonal antibody coating the walls of a reaction tube. Captured virus was then heat denatured to release virion RNA into a universal buffer, permitting reverse transcriptase-mediated cDNA synthesis to be followed directly by Taq polymerase-directed PCR without isolation of viral RNA or primary cDNA reaction products.

A detailed description of the methodology (Jansen et al., in preparation) and its application to the study of the molecular epidemiology of HAV follows.

Antigen-Capture/Polymerase Chain Reaction Procedure Sterile 1.5 ml polypropylene microcentrifuge tubes were coated with a 1:300 dilution of anti-HAV murine ascitic fluid, then "blocked" with 1% bovine serum albumin as described for immunoaffinity hybridization (Jansen et al., 1985). The antibody-coated tubes were washed 3 times with 300 ul of phosphate buffered saline (PBS) containing 0.05% Tween-80 and 0.02% sodium azide, loaded with virus-containing samples (50 to 80 ul), and held overnight at 4 °C. Tubes were then washed 6 times with 300 ul of 20 mM Tris, pH 8.4, 75 mM KCl, and 2.5 mM $MgCl_2$, with appropriate measures taken to avoid cross contamination (disposable Combitips, Eppendorf). Universal buffer (80 ul, 25 mM Tris, pH 8.4, 75 mM KCl, 2.5 mM $MgCl_2$, and 0.25 mM each of dATP, dCTP, dGTP and TTP) was added to each virus-coated tube along with 5 ul (100 pmol) of negative-strand primer (see legend to Figure 6). Following denaturation of virus at 95°C for 5 minutes and cooling to 42°C, reverse transcriptase was added (2 units in 5 ul, Life Sciences). Reaction mixtures were held at 42°C for 30-60 minutes, after which 5 ul (100 pmol) of positive-strand primer and 5 ul containing 2 units of Taq polymerase (Cetus-Perkin Elmer) and 20 ug of gelatin were added. Reaction mixes were overlaid with mineral oil and subjected to 30 automated cycles of denaturation at 95°C for 15 seconds, annealing at 37°C for 5 seconds, and extension at 70°C for 1 minute, with an additional 5 minute incubation at 70°C during the final extension. Reaction products were ethanol precipitated and resuspended in 15 to 30 ul of sterile H_2O.

The procedure was applied to fecal samples obtained from an owl monkey inoculated with cell culture-adapted HAV (see Report Number 2; Lemon et al., 1989), human volunteers undergoing experimental infection with HAV (Boggs et al., 1970), a patient developing hepatitis A in North Carolina, or soldiers involved in military outbreaks of hepatitis A in Kansas and the Federal Republic of Germany during 1982 (Lednar et al., 1985). Additional materials (including the Greek specimens AG11, AG5978 and AG6014, originally collected by G. Papaevangelou) were provided by G. Siegl through the HAV Strain Bank of the World Health Organization's Programme for Vaccine Development. Fecal samples were tested as clarified 5-10% suspensions.

Analysis of AC/PCR Amplification Products. A 30% aliquot of the amplified products was fractionated through 3% NuSieve GTG/1% SeaKem ME (FMC) agarose gels in Tris-Acetate/EDTA buffer, and visualized by staining with ethidium bromide. For oligonucleotide hybridization, amplified DNA products were transferred to nitrocellulose membranes by conventional methods. Prehybridization was carried out at 37°C in 6X SSC (1X SSC is 0.15 M NaCl/0.015 M sodium citrate), 5X Denhardt's solution (1X Denhardt's is 0.02% each of bovine serum albumen, Ficoll, and polyvinylpyrrolidone), 0.5% SDS, 0.05% sodium pyrophosphate, and 100 ug/ml calf thymus DNA. Hybridization was for 20 hours at 37°C in a similar buffer containing 1X Denhardt's solution and 0.2 pmol/ml (5 uCi/pmol) of an HAV-specific oligonucleotide (see legend to Figure 6), 5' end-labelled with T4 polynucleotide kinase (Promega Biotek).

Blots were washed 5 times at room temperature in 6X SSC, 0.05% sodium pyrophosphate, and exposed to XAR-5 film (Eastman Kodak) for 16 hours.

Nucleotide Sequence of AC/PCR Products. AC/PCR products were purified from 3% NuSieve GTG agarose gels (FMC) by phenol extraction and ethanol precipitation, and sequenced by the dideoxy method using Sequenase (USBC). DNA (10 to 100 ng), 5X buffer, and 1-2 pmol of [^{32}P]-end-labelled primer were diluted to 18 ul, heated to 97°C for 3 minutes, and cooled quickly on ice. While at 4°C, 4 ul were dispensed into each of 4 microplate wells containing the dideoxy-/deoxynucleotide triphosphate and Sequenase mixes (2 ul each). Reactions were mixed by brief centrifugation, immediately placed at 37°C for 10 minutes, then stopped by addition of 4 ul of stop mix. After heating at 80°C for 10 minutes, 2 ul were loaded onto 6% polyacrylamide/7 M urea gels. Following electrophoresis, gels were exposed to XAR-5 film (Kodak) for 1 to 4 days.

Sensitivity of AC/PCR   To evaluate the sensitivity of AC/PCR, we compared its ability to detect viral nucleic acid with a conventional PCR method involving the enzymatic amplification of cDNA made from HAV RNA that had been prepared by phenol/chloroform extraction following incubation of virus with SDS and proteinase K. Serial dilutions of a suspension of feces collected from an experimentally infected owl monkey were tested in parallel by both methods (Figure 6). A region in the viral genome 206 nucleotides in length and encoding capsid protein VP3 near its carboxy terminus was selected for amplification by PCR (Figure 6A). In replicate experiments, both AC/PCR and the conventional PCR method yielded a single amplified reaction product of the expected length, confirmed to contain the appropriate HAV sequence by Southern hybridization (Figure 6B and 6C). The end-point dilutions were similar for both methods, indicating that heat-denatured, affinity-isolated virus provided template of sufficient purity for subsequent polymerase reactions.

Since the fecal specimen tested in this experiment was collected from an owl monkey that had been inoculated with a cell culture-adapted HAV variant (see Report Number 2; Lemon et al., 1989), we were able to compare the detection limits of AC/PCR with the infectious titer determined by an in vitro radioimmunofocus assay ($10^{7.3}$ radioimmunofocus-forming units (RFU)/g of the original fecal sample). With serial dilutions of the fecal suspension down to that containing as little as 0.47 RFU in the tested sample, AC/PCR resulted in progressively lesser amounts of the amplified reaction product (Figure 6B). A weak signal was detected at 0.047 RFU, while an isolated positive result was obtained at 0.0047 RFU. PCR amplification of cDNA derived from phenol/chloroform-extracted viral RNA appeared similarly quantitative with respect to input virus, with reaction products consistently detected down to 0.047 RFU (Figure 6C). Previous experiments in our laboratory have suggested that the particle/infectivity ratio of cell culture-adapted HAV is approximately 60:1 (see Report Number 2; Jansen et al., 1988); thus, the limits of detection by both techniques is in the range of 3 to 30 virus particles.

Specificity of AC/PCR   We assessed the contribution of immunoaffinity purification of virus to the overall specificity of AC/PCR. HAV-containing samples were placed into reaction tubes coated with monoclonal antibodies to HAV, poliovirus type 1 (PV1), or respiratory syncytial virus (RSV) for assay by AC/PCR. Strong hybridization signals were obtained with products from reaction tubes coated with anti-HAV monoclonal antibody, while residual virus present in reaction vessels coated with nonspecific antibodies led only occasionally to low levels of amplified HAV reaction products detectable by Southern hybridization. Thus, the anti-HAV monoclonal antibody provided a high level of specificity, despite the sensitivity of the subsequent PCR amplification procedure.

Application of AC/PCR: Comparison with Conventional Methods for Detection of HAV   Fecal specimens from 21 acutely infected American soldiers were examined for the presence of HAV by means of AC/PCR. These specimens had been collected during the first week of illness from IgM anti-HAV positive soldiers involved in a common source outbreak of hepatitis A which occurred during a field exercise at Grafenwohr, in the Federal Republic of Germany, during the summer of 1982 (see relevant EPICON report; Lednar et al., 1985). Seventeen of 21 (81%) individual fecal specimens yielded reaction products (206 bp) that were visualized in an ethidium bromide-stained agarose gel. Further analysis by Southern hybridization revealed 2 additional positive samples (data not shown), bringing the total number of HAV-positive specimens to 19 (90%). The sensitivity of AC/PCR was thus significantly greater than solid-phase radioimmunoassay and immunoaffinity cDNA/RNA hybridization (Jansen et al., 1985), two methods commonly used for detection of HAV in clinical samples. Of 18 fecal specimens that were positive for HAV by AC/PCR, only 13 were positive by cDNA-RNA hybridization. Ten of these specimens contained HAV antigen detectable by solid-phase radioimmunoassay. In general, there was a quantitative relationship between the cDNA-RNA hybridization blot intensity, radioimmunoassay sample/negative control (S/N) ratio, and the quantity of PCR-amplified product obtained from each positive fecal sample (not shown). These results, along with those displayed in Figure 6B, suggest that AC/PCR is at least semi-quantitative under the conditions employed.

Molecular Epidemiology of HAV   Reaction products amplified by AC/PCR from clinical samples (or cell culture materials) containing HAV obtained in different epidemiologic settings were sequenced by extension of labelled primers in the presence of dideoxynucleotides. To compare HAV strains, we analyzed colinear sequences of each virus derived from two different genomic regions. These regions spanned nucleotides 2056 to 2208 (carboxy terminal region of VP3), and 3020 to 3191 (VP1/2A junction region) in the viral genome. The 325 nucleotide bases thus included in this analysis represent approximately 5% of the viral genome. We found no differences in the sequence of AC/PCR reaction products derived from cell culture-adapted HM175, CR326 or HAS15 strains of HAV, and the sequences of these viruses reported previously from molecularly cloned cDNA (Cohen et al., 1987a; Linemeyer et al., 1985; Ovchinikov et al., 1985), demonstrating the accuracy of sequence data derived from viral RNA by AC/PCR. The AC/PCR-derived sequence of strain MBB fecal material differed from the reported cDNA-derived sequence at a single base position (Paul et al, 1987). However, substantial differences (up to 9.8%

17

nucleotide nonidentity) were found between AC/PCR reaction products of HAV strains obtained in different epidemiologic settings. These results allowed construction of a dendrogram showing genetic relatedness among these strains (Figure 7). Similar results were obtained by independent comparisons within each genomic region (VP3 and VP1/2A), indicating that the sample size was sufficient for reliable measures of relatedness. Virus strains could be placed into two major groups, one consisting of viruses collected in Australia, North Africa and Greece, and the other comprised of viruses recovered in North America, Northern Europe, and China (Figure 7). Relatedness within these groups was 93.5% or greater at the nucleotide level (often greater than 95%), while the relatedness between viruses in different groups was less than 93.2% (often less than 92%). Most differences occurred at the third-base position within codons. The predicted amino acid sequence differed among these viruses at only one residue: residue 297 of capsid protein VP1, which was lysine in strains from Australia, North Africa and Greece, but arginine in the other HAV strains studied.

Viruses present in fecal samples collected from three soldiers (GR-1, GR-7 and GR-CL) involved in the 1982 Grafenwohr outbreak of hepatitis A in the Federal Republic of Germany shared a common nucleotide sequence, as might be expected. Similarly, fecal specimens collected from three cases of endemic hepatitis A occurring in central Greece over a 5 month period during 1983 (AG11, AG5978, and AG6014) contained viruses that were identical or differed at a single base position, suggesting the presence of a single virus circulating in Greece at that time. However, the sequences of the viruses recovered in Greece and in Germany differed from each other by 9.8%. As this difference is as great as that between any two strains of human HAV, these data indicate the existence of distinct strains of HAV in Northern and Southern Europe during 1982-1983 and suggest a new view of the epidemiology of HAV in human populations.

Of particular interest was the fact that fecal specimen LV-BE contained virus with a sequence identical to the virus causing the German epidemic. LV-BE was collected from an American soldier involved in an epidemic of hepatitis A which occurred within the military prison at Fort Leavenworth, Kansas during the summer of 1982 (see relevant EPICON report; Lednar et al., 1985). The fact that the sequence of this virus was identical to that causing a simultaneous outbreak among American soldiers in Europe establishes a clear but previously unrecognized epidemiologic link between these two HAV epidemics. This result dramatically underscores the power of this approach to studying the epidemiology of HAV at the molecular level.

Also surprising was the fact that HAS-15 and LCDC viruses (obtained from the Laboratory for the Center for Disease Control, Ottawa, Canada) shared identical sequences. This result may indicate very close relatedness two among diverse isolates (HAS-15 was recovered near Phoenix, AZ, while LCDC was recovered from human feces collected in China). However, several other cell culture derived "isolates" from other laboratories appear to be contaminants derived from prototype laboratory strains and not original isolations of HAV. In such cases, we have compared the nucleotide sequence of the "isolate" with that of virus in fecal material. An example is the KMW-1 strain of HAV: the

18

sequence of this virus when studied from feces is markedly different from that of the related cell culture-adapted isolate (obtained from G. Siegl, Bern, Switzerland), which is itself identical to the MBB strain. In this case it is certain that the KMW-1 "isolate" is an MBB contaminant, as MBB virus was also under culture in this laboratory. Since the LCDC sequence was derived from virus propagated in cell culture, a firm conclusion concerning the relatedness of LCDC and HAS-15 must await the testing of LCDC virus present in fecal samples.

Cell culture-adapted CLF strain HAV (obtained from G. Siegl) was also identical in sequence to MBB virus. We were, however, unable to successfully amplify cDNA from CLF fecal material despite several attempts with two different primer sets. These data must raise concerns about the possibility that CLF virus, which has been utilized for development of an inactivated HAV vaccine, may also be an MBB contaminant.

IV. Partial genomic sequence of the PA21 strain of HAV recovered from New World Panamanian owl monkeys.

In 1980, during collaborative studies between the WRAIR and the Gorgas Memorial Institute in Panama, an HAV strain (PA21) was recovered from a feral owl monkey shortly after its capture and admission to a primate holding facility in Panama (Lemon et al., 1982; Binn et al., 1984). Serologic testing of other owl monkeys demonstrated wide-spread infection with HAV within the colony during the preceding years, with most wild-caught animals seroconverting within months of capture. Virus recovered from infected monkeys was found to be antigenically similar to human HAV by quantitative in vitro neutralization assays (Lemon and Binn, 1983), and in preliminary studies with monoclonal antibodies (Lemon et al., 1987). Yet, slot-blot cDNA-RNA hybridization with probes derived from human HAV has suggested that the PA21 virus was distinct from human HAV isolates (Lemon et al., 1987). We have further characterized the PA21 virus, in an effort to determine the basis of its genetic diversity and how this might relate to its highly conserved antigenic structure.

RNA was isolated from gradient-purified PA21 virus. Random oligonucleotide primers were used to generate double-stranded cDNA by the method of Gubler and Hoffman (Brown et al., 1989). The cDNA was blunt-ended with T4 DNA polymerase and ligated to EcoRl linkers using T4 DNA ligase, followed by digestion with EcoRl. The cDNA was ligated into the EcoRl site of the plasmid vector pTZ18R (U.S. Biochemical) and the cDNA-vector hybrids were used to transform competent E. coli NM522 cells. Ampicillin resistant clones were screened by in situ colony hybridization using [^{32}P]-labelled randomly primed cDNA prepared from PA21 RNA. Single-stranded DNA was prepared from positive clones and sequenced using the dideoxy chain termination method of Sanger.

Overlapping clones spanning part of the 5' noncoding region, the complete P1 region and the 5' portion of the P2 region of PA21 were sequenced completely, and the derived sequence compared with known sequences of human HAV strains. Additional sequence was obtained from 3' noncoding and the 3'

terminus of the P3 genomic region. The 5' noncoding, P2, P3 and 3' noncoding regions were found to have 89.2%, 79.6%, 84.4%, and 93.7% nucleotide identity with respect to the human HM175 virus (Brown et al., 1989). The G+C content of PA21 RNA was found to be 39.5%, only slightly higher than that of other HAV strains. The codon usage was unremarkable except for the leucine TTA codon which is used by PA21 with less than half the frequency of the human HAV strains (5/63 vs 13/63 for HM175). The typical HAV bias against the CG nucleotide pair was present (frequency of use 0.4%).

The P1 region of PA21 genomic RNA was found to comprise 2373 nucleotides and to encode 791 amino acids. The nucleotide difference between PA21 and sequenced human HAV strains was approximately 83% in the capsid-encoding region. The region encoding VP1 (Figure 8) demonstrated the greatest divergence (78.4% - 81.3% identity with 6 human HAV strains), while the VP3 encoding region, as in comparisons of other picornaviruses, was the most conserved (84.7% - 86.0% identity). The majority of the nucleotide changes occur in the third codon position (343 of 398, in comparison with HM175), while differences present in the first or second positions often do not lead to a change in the amino acid. Thus, the amino acid sequence of the capsid proteins is largely conserved between the simian and human viruses.

Overall, the PA21 P1 genomic region sequence indicates that its capsid differs at only 23 (HM175 and MBB) to 31 (HAS15) amino acid residues when compared with the capsids of human HAV strains. Most of these changes (16 out of 23) are conservative in nature. Although the nucleotide changes are distributed randomly across the P1 region (except for the region encoding the carboxy terminus of VP1, at the 3' end of the P1 region), amino acid replacements are clustered near the amino termini of VP2 and VP1, and the carboxyl terminus of VP1 (for details, see Brown et al., 1989). Of the 31 carboxy terminal amino acids of VP1, eight are different from HM175 virus.

CONCLUSIONS

Genomic-length p16 cDNA clone   HM175 cDNA clones have been assembled into a genomic length construct containing the p16 HM175 consensus sequence. Flanking dC-dG tails remaining from the original homopolymeric RNA/cDNA cloning procedure were removed, and the full-length construct has been inserted into the vector pGEM3 immediately downstream of the SP6 transcriptional start site. Studies defining the infectivity of this plasmid (either as DNA or RNA) remain in progress, but it is expected that this genomic length construct will be of considerable value in further defining the critical mutations associated with adaptation of p16 HM175 to growth in cell culture. Furthermore, this construct may provide the means of attempting alternate approaches to attenuation of the virus, as deletion of regions of the 5' noncoding region of wild-type poliovirus has been shown to result in substantial attenuation (Nomoto et al., 1987). Such attenuated mutants should be extremely stable with little chance of reversion to virulence.

<u>Cytopathic HM175 HAV variants</u> Currently there is very little
understanding of the mechanism underlying the cytopathic effect observed with
some cell culture-adapted variants of HAV. In general, these cytopathic HAVs
appear to be capable of relatively rapid, efficient replication in cell
culture when compared with noncytopathic but otherwise cell culture-adapted
HAV variants (Table 1) (Anderson, 1987; Cromeans et al., 1987; Nasser and
Metcalf, 1987). Unlike poliovirus, however, which induces a cytopathic effect
in association with a shutdown of host cell protein synthesis, cytopathic HAV
variants do not appear to interfere with host cell macromolecular synthesis.
Inasmuch as cytopathic HAVs are variants that have become uniquely well
adapted to growth in cell culture, it is probable that the cytopathic effect
reflects a quantitative change in the ability of the virus to replicate in
vitro rather than a qualitative change in the replication strategy of the
virus. If this is the case, it is likely that multiple mutations play a role
in determining the cytopathic phenotype, just as multiple mutations appear to
be important in the primary adaptation of virus to growth in vitro (Jansen et
al., 1988; Cohen et al., 1987b). From a practical point of view, the in vitro
growth characteristics of some of these cytopathic variants (e.g., the 18f
variant) may make them excellent candidates for development of inactivated
vaccines or the production of viral antigen for diagnostic tests.

We defined capsid mutations present in two rapidly replicating,
cytopathic HM175 virus variants. We demonstrated that an antigenically
altered K24F2⁻ virus variant was spontaneously selected during passage of
cells that were persistently infected with HM175 virus (in the absence of
antibody pressure). This variant (43c virus) grew relatively rapidly and was
cytopathic in both FRhK-4 and BS-C-1 cells; it had amino acid substitutions at
residues 3-070, 1-197, and 1-276. The mutation at 3-070 is most likely
responsible for antigenic variation in 43c virus, as mutation at this residue
has been associated with neutralization escape from K24F2 in viruses
specifically selected for resistance to antibody (Ping et al., 1988).
However, a role for the other capsid mutations in the altered antigenicity of
43c virus (particularly the mutation at 1-276) has yet to be excluded. A
second cytopathic HM175 variant was isolated from the same persistently
infected cell cultures; this variant (18f virus) had mutations at similar
sites in the capsid proteins (residues 3-091 and 1-271) but was antigenically
indistinguishable from the parent HM175 virus in solid-phase immunoassays and
virus neutralization tests with monoclonal antibodies.

Although several explanations are possible for the emergence of an
antigenically altered virus during persistent HAV infection, the data
presented here suggest that the ability of HAV to replicate rapidly or induce
a cytopathic effect in vitro is not due to a unique capsid mutation.
Mutations in the region of VP3 residues 3-070 to 3-091 and VP1 residues 1-271
to 1-276 may influence replication of the virus during persistent infection
and serial passage in vitro, as they were present in both 43c and 18f rapidly
replicating, cytopathic variants, but the extent to which they do so remains
uncertain. It is likely that mutations elsewhere in the genome play important
roles in determining the cytopathic phenotype. Mutations in the 5' noncoding
region and in the P2 (proteins 2B and 2C) and P3 ($3D_{pol}$) genomic regions
appear to be important in the initial adaptation of virus to growth in cell

culture (Cohen et al., 1987b; Jansen et al., 1988), and thus may also play dominant roles in the evolution of virus to a rapidly replicating cytopathic phenotype. Further sequencing efforts and the construction of infectious cDNA from cytopathic HAV variants will be necessary to resolve this issue.

Sequence diversity among HAV strains With the testing of new HAV vaccines by the U.S. Army and other agencies, there is a growing need for a rapid and sensitive method capable of detecting and distinguishing specific strains of HAV in human samples. Our data demonstrate the utility of AC/PCR as a diagnostic procedure and support its use in characterizing the molecular epidemiology of viruses such as HAV. Important benefits derive from combining the specificity of an immunoaffinity reaction with the sensitivity of PCR for virus detection, including simple approaches to confirming the specificity of amplified DNA products. Of practical significance, the AC/PCR assay involves few manipulations and is technically relatively simple, features that should reduce the risk of sample contamination. It is applicable to the testing of substantial numbers of specimens in epidemiologic or clinical studies, including future HAV vaccine trials.

Rico-Hesse and coworkers (Rico-Hesse et al., 1987) have demonstrated that contemporary isolates of single poliovirus serotypes commonly share less than 85% nucleotide identity. We found considerably greater genetic relatedness among human HAV isolates collected worldwide (Figure 7), although HAV and polioviruses share many other common genomic features. Remarkably, the MS-1 and NC-1 strains of HAV (collected 25 years apart) have nucleotide sequences that are 96.6% identical and show no amino acid changes within the regions sequenced. While conservation of the primary structure of HAV capsid proteins may reflect structural constraints imposed on the evolutionary process, considerable divergence in the amino acid sequence of the carboxy terminus of VP1 has been found recently in the PA21 simian strain of HAV (Brown et al., 1989). Thus there must other reasons for the high level of conservation that we found among human HAV strains.

Simian PA21 HAV Cloning and sequencing studies indicate that PA21 virus represents a unique genotype of HAV and suggest the existence of an ecologically isolated niche for HAV among feral owl monkeys.

TABLES AND FIGURES

TABLE 1.   Cytopathic Effect and Intracellular and Extracellular HAV Antigen
Accumulation Following Infection of FRhK-4 Cells at Low Multiplicities[*].

Virus	Cytopathic Effect (day PI)							HAV Antigen (cpm)		
	0	2	4	6	7	9		Day PI	Cells	Media
p16 HM175	0	0	0	0	0	0		9	454	76
43c	0	0	0	+(+)	++	nd		7	2145	72
18f	0	0	0	(+)	+	nd		7	3623	820

[*]Parallel cultures of FRhK-4 cells were established in 25 cm^2 flasks, and
inoculated with 580 RFU 43c virus, 460 RFU 18f virus, or 1500 RFU p16 virus.
Cytopathic effects were scored as: 0 - none, + - 10% cells detached, ++ - 25%
cells detached; PI - postinoculation, nd - not done.

TABLE 2. Mutations in P1 Genomic Region of HM175 Virus Variants.

| Virus | Nucleotide | | Amino Acid | |
	Position	Substitution[*]	Residue	Replacement[*]
p16 HM175	964	A → G	2-054[**]	K → R
	1742	G → A		
43c	964	A → G	2-054	K → R
	1678	A → C	3-070	D → A
	1742	G → A		
	2797	A → G	1-197	N → S
	3033	A → G	1-276	M → V
18f	964	A → G	2-054	K → R
	1741	C → A	3-091	T → K
	1742	G → A		
	2684	C → U		
	3018	U → C	1-271	S → P

[*]From wild-type sequence (Cohen et al., 1987a).
[**]By convention, residue "2-054" is residue 54 of capsid protein VP2.

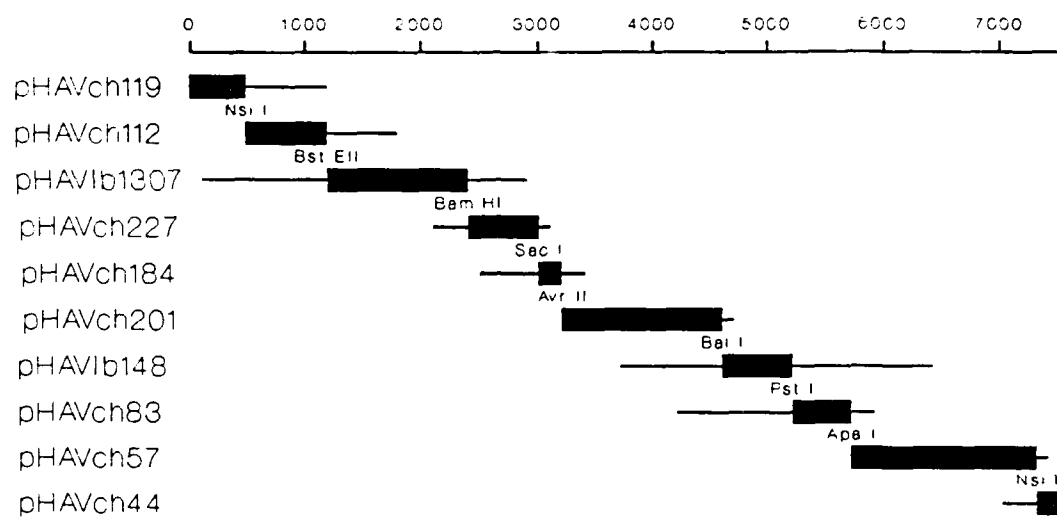FIGURE 1. Map of the genomic-length p16 HM175 construct pHAV$_{ch}$p16: restriction sites employed for individual ligation reactions and contributing cDNA plasmid clones are shown.
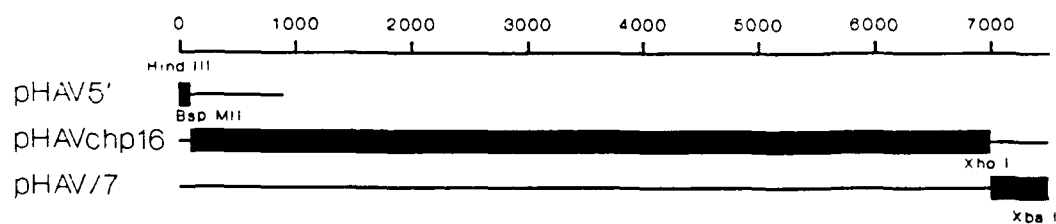
FIGURE 2. Final construction of pHAV/p16: ligation of fragments from pHAV5', pHAV$_{ch}$p16, and pHAV/7 to create a genomic-length p16 HM175 sequence inserted between HindIII and XbaI sites of the plasmid vector pGEM3.
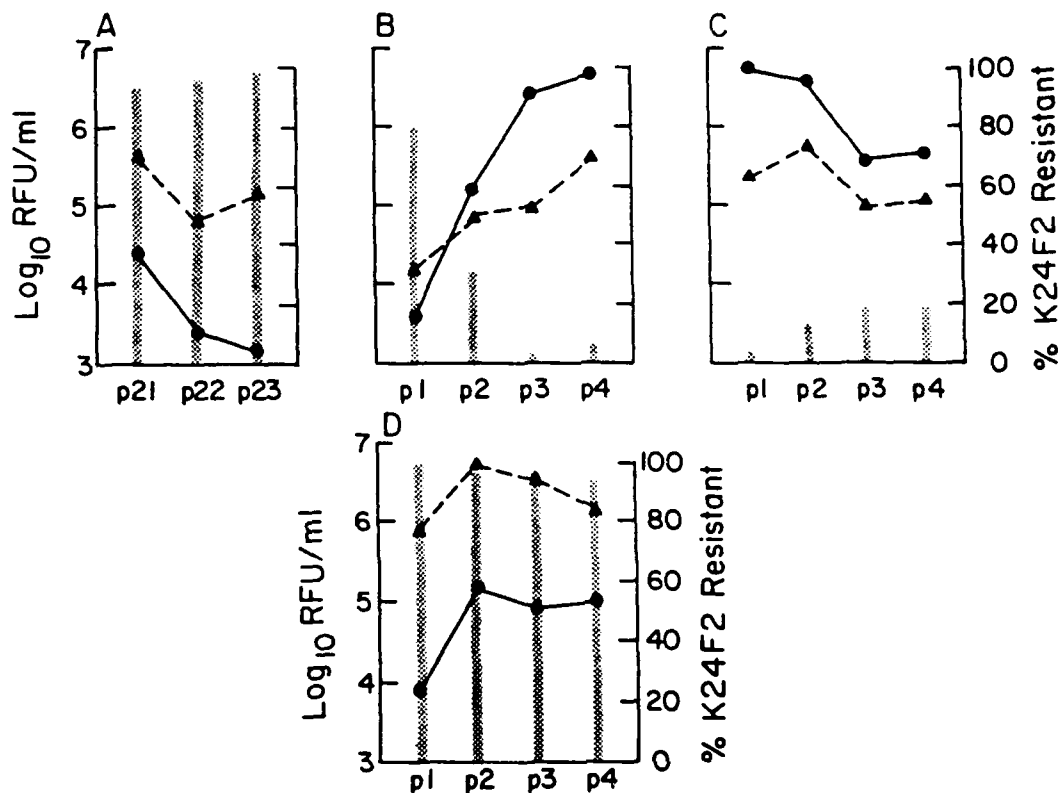
FIGURE 3. K24F2⁻ (▲---▲) and K24F2⁺ (●——●) virus populations in
pHM175 virus harvests following persistent infection in BS-C-1 cells (panels A
and C), and serial passage in FRhK-4 cells (panel B) or BS-C-1 cells (panel
D). Bars (▓) represent the percent of each virus harvest that had K24F2⁻
phenotype. Panel A represents the last three passages (p21-p23) of pHM175 in
the persistently infected BS-C-1 cell line (Cromeans et al., 1987). In panels
B and D, virus derived from the BS-C-1 cell p23 persistent infection harvest
(panel A) was passed in serial fashion with disruption of cells. In panel C,
virus recovered from the p4 serial FRhK-4 passage harvest (panel B) was used
to reestablish persistent infection in BS-C-1 cells.
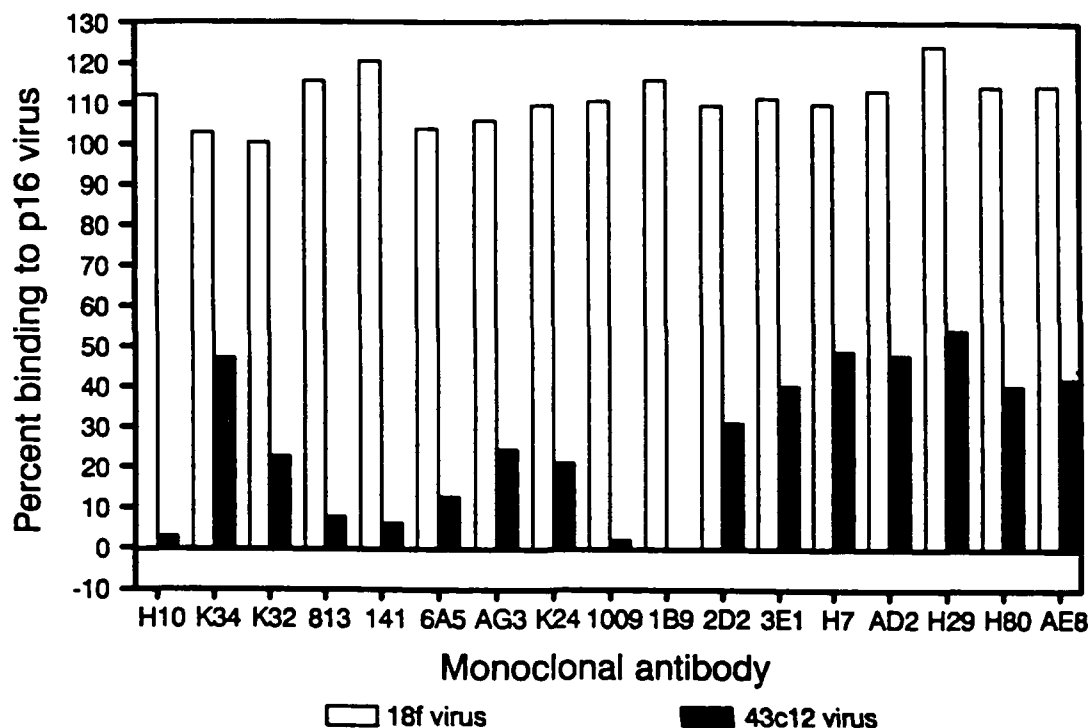
FIGURE 4. Binding of murine monoclonal antibodies to 18f (□) and 43c
(■) virus variants, determined by an indirect radioimmunoassay. Antibody
binding to 18f or 43c is expressed as the percentage of binding to equivalent
amounts of the parental p16 HM175 virus in companion assays. Virus
concentrations tested in the assays were standardized by cDNA-RNA
hybridization.
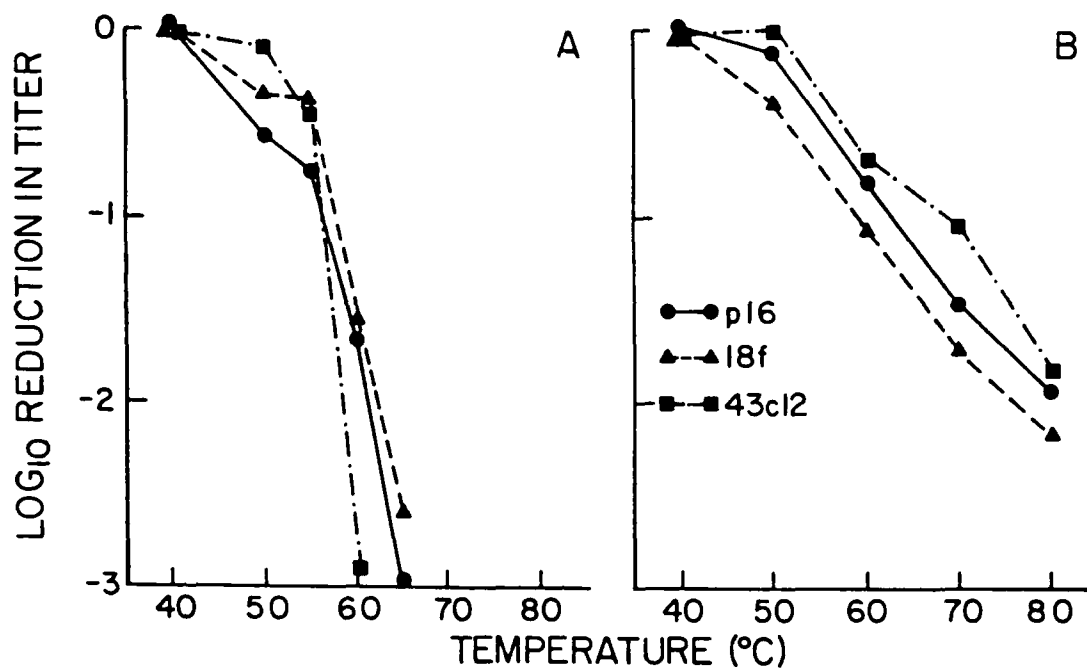
FIGURE 5. Thermal stabilities of infectious noncytopathic p16 HM175 virus (•——•), and the rapidly replicating, cytopathic 18f (▲---▲) and 43c (■.—.■) viruses. Crude cell lysates were heated at the indicated temperature for 10 min in the absence (panel A) or presence (panel B) of additional 1 M Mg^{++}, and subsequently assayed by radioimmunofocus assay for infectious virus. Virus incubated at 40 °C was unchanged in titer from control virus held at 0 °C.
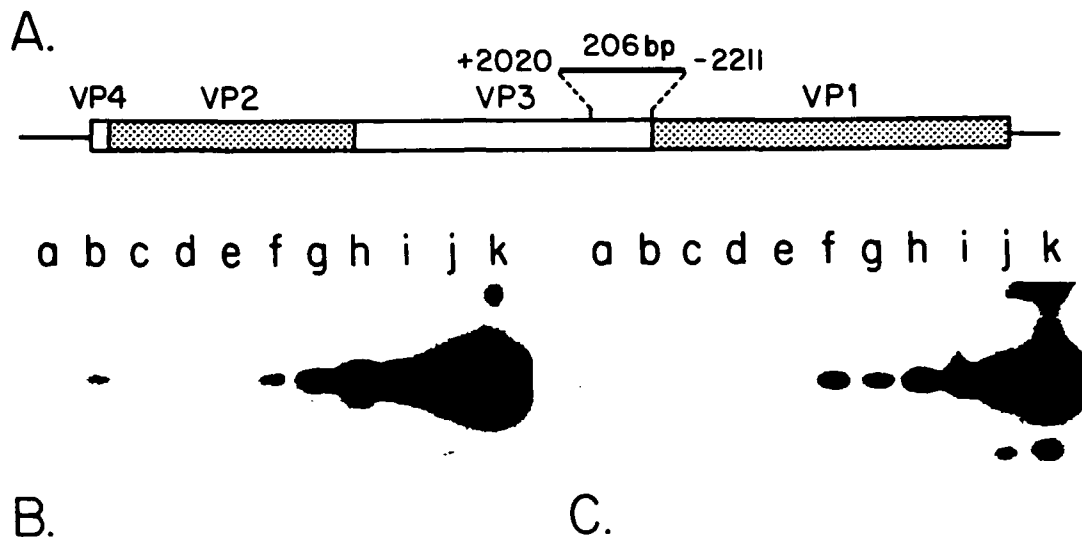
FIGURE 6. AC/PCR detection of HAV RNA encoding the carboxy terminal region of capsid protein VP3. (a) The P1 (capsid protein-encoding) region of the HAV genome showing locations of PCR positive-strand primer HAV+2020 (5'-ACAGGTATACAAAGTCAG) and negative-strand primer HAV-2211 (5'-CTCCAGAATGATCTCC), designated according to most 5' genomic bases. (b,c) Southern hybridizations of agarose gel-fractionated AC/PCR (b) and conventional PCR (c) products made from primate fecal suspensions with the primers above. The end-labelled oligonucleotide used for hybridization was HAV+2189 (5'-TATGGATGTTACTACACA). Lanes "b-k" contain reaction products made from serial half-log dilutions of a fecal sample collected from an owl monkey infected with a cell culture-adapted variant of HM175 virus. Titrations of this fecal sample in cell culture indicated that lane "b" contained products made from 0.0047 radioimmunofocus-forming units (RFU) of infectious virus (10); "c" 0.015 RFU; "d" 0.047 RFU; "e" 0.15 RFU; "f" 0.47 RFU, "g" 1.5 RFU, "h" 4.7 RFU, "i" 15 RFU, "j" 47 RFU, and "k" 150 RFU. Lane "a" contains PCR reaction products made from PBS only. For AC/PCR detection of the genomic region spanning the carboxy terminus of VP1 and the amino terminus of protein 2A, the primers utilized were HAV+2984 (5'- TCCCAGAGCTCCATTGAA) and HAV-3265 (5'- CATTATTTCATGCTCCTCAG).

KMW-1	Switzerland	1979
NC-1	USA, NC	1989
LCDC*	China	1984
HAS-15*	USA, AZ	1979
FR-AL*	USA, AK	1976
LV-BE	USA, KS	1982
GR-1	W. Germany	1982
GR-7/CL	W. Germany	1982
CR-326*	Costa Rica	1966
MS-1	USA, NY	1964
S85-1	China	1985
MBB	N. Africa	1978
HM175*	Australia	1976
Ag11	Greece	1983
Ag5978	Greece	1983
Ag6014	Greece	1983

% IDENTITY (scale: 100, 98, 96, 94, 92, 90)
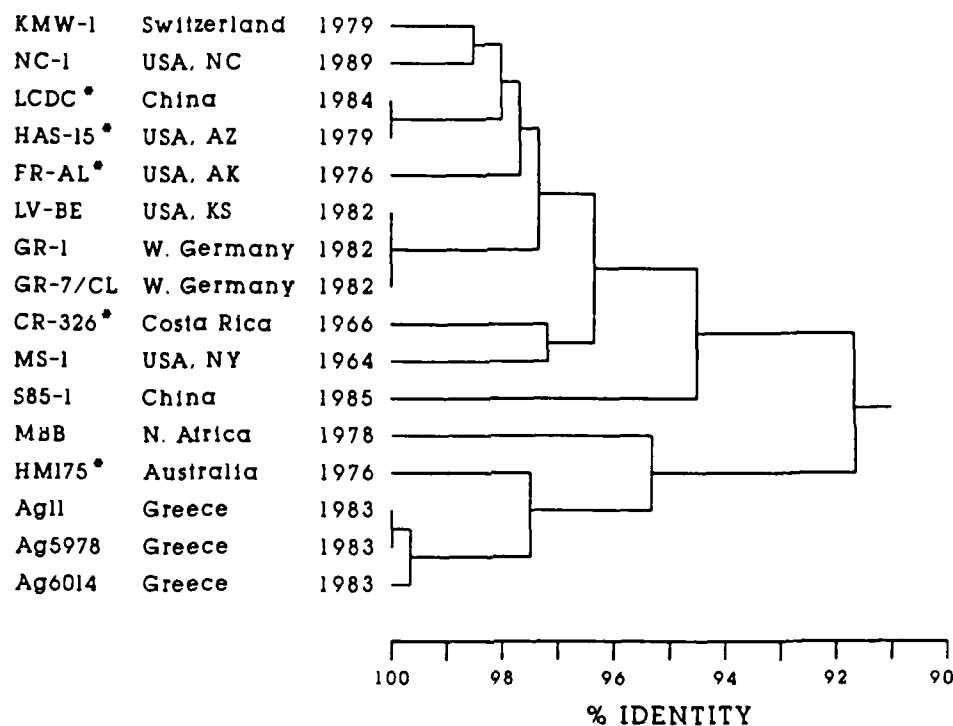
FIGURE 7. Dendrogram showing relatedness between nucleotide sequences of HAV strains from epidemiologically diverse sources. Differences between colinear sequences spanning bases 2056 to 2208 and 3020 to 3191 (325 total) were combined for this analysis; similar results were independently obtained in both genomic regions. The approximate degree of nucleotide identity between any two strains is represented by the distance from the left of the diagram to the first common node. The dendrogram was constructed by comparative subset averaging of percent identity data. (* from cell culture)

31

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     -----A---------------T--T--A--A--A-----T---G-----------------T--C---------T--A-----CA----A---T-G-----A-----T---AG----------
2208 GTTGGGGATGATTCTGGAGGCTTCTCTACCACTGTTTCAACAAAACAGAATGTTCCAGACCCTCAAGTTGGCATTACAACAGTGAAGGATCTTAAAGGTAGAGCAAACCAAGGGAAAATG
     V  G  D  D  S  G  G  F  S  T  T  V  S  T  K  Q  N  V  P  D  P  Q  V  G  I  T  T  V  K  D  L  K  G  R  A  N  Q  G  K  M
     .  .  .  .  .  .  .  .  .  .  .  E  .  .  .  .  .  .  .  .  .  .  .  .  .  .  M  .  .  .  .  K  .  .  R  .  .  .

         --------A--AG-A-----A-----G-----------A--A----------------A-----------A--------A--T--T--------A--------A--C--------A--A
2328 GATGTTTCGGGTATCCAAGCTCCTGTAGGAGCTATCACTACCATTGAGGATCCAGTTTTGGCAAAGAAAGTGCCTGAGACCTTCCCAGAATTGAAGCCTGGAGAGTCAAGACATACTTCT
     D  V  S  G  I  Q  A  P  V  G  A  I  T  T  I  E  D  P  V  L  A  K  K  V  P  E  T  F  P  E  L  K  P  G  E  S  R  H  T  S
     .  .  .  .  V  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .

         -----------C--C-----G---------A--G-----------G--C--T--------C-----A-----T-----------A--------A--C-----T-----C-----------
2448 GATCATATGTCTATTTACAAATTTATGGGCAGATCTCATTTCTTATGTACATTTACATTTAATTCTAATAACAAAGAGTACACTTTTCCTATCACTTTGTCATCAACTTCTAATCCTCCT
     D  H  M  S  I  Y  K  F  M  G  R  S  H  F  L  C  T  F  T  F  N  S  N  N  K  E  Y  T  F  P  I  T  L  S  S  T  S  N  P  P

         -----T-----A-----A-----G---------C--T-G-------T-G-----A--G--T--A---C-------T-----T--A--A--A--------A-----C-----C-----C---
2568 CATGGATTGCCTTCAACTCTGAGATGGTTTTTTAACCTTTTTCAGCTTTATAGGGGTCCCTTGGATTTGACAATAATTATAACTGGGGCTACTGATGTTGATGGAATGGCTTGGTTTACT
     H  G  L  P  S  T  L  R  W  F  F  N  L  F  Q  L  Y  R  G  P  L  D  L  T  I  I  I  T  G  A  T  D  V  D  G  M  A  W  F  T
     .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .

         --A--A--A--TC-T--C--T-----T--T-----A-----------A---T-G---------C-----A--T--------A-----C--A-----C--A--G-------G----------
2688 CCCGTTGGGTTAGCAGTAGATACCCCATGGGTTGAGAAGGAGTCTGCTCTTTCTATTGATTACAAGACAGCTCTTGGTGCTGTTAGGTTTAATACTAGAAGAACAGGAAACATTCAGATT
     P  V  G  L  A  V  D  T  P  W  V  E  K  E  S  A  L  S  I  D  Y  K  T  A  L  G  A  V  R  F  N  T  R  R  T  G  N  I  Q  I
     .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .

         --A--A--A-----T--T---T-A---------G--T--A-----------TT-G--T-----G---------T--A-------T-G--T--T-----G------------------T--T---
2808 AGGTTGCCCTGGTACTCCTATCTTTATGCTGTCTCAGGGGCACTGGATGGGCTTGGAGACAAAACAGATTCAACTTTTGGACTTGTCTCCATTCAAATTGCAAATTACAATCACTCAGAT
     R  L  P  W  Y  S  Y  L  Y  A  V  S  G  A  L  D  G  L  G  D  K  T  D  S  T  F  G  L  V  S  I  Q  I  A  N  Y  N  H  S  D
     .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .

         -----C-----------------T---------C--A-----A--A--------------C-----T--A-----CT-A-----C---T-A--CA-T---T--------AGC--------A
2928 GAATATTTGTCTTTTAGTTGTTACTTGTCTGTGACTGAACAGTCTGAGTTTTATTTTCCTAGAGCCACCTTTGAATACCAATGCTATGATGTCATCAGAAACAATGATGGATAGAATTGCT
     E  Y  L  S  F  S  C  Y  L  S  V  T  E  Q  S  E  F  Y  F  P  R  A  P  L  N  T  N  A  M  M  S  S  E  T  M  M  D  R  I  A
     .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  S  .  .  .  L  .  T  .  S  .  .  S  .  .  .

     GC---A--CT-G--G--A-----G---------A--T-A--G--A---AAA-G------G
3048 CTTGGTGATCTTGAATCCTCAGTTGATGATCCTCGAACTGAAGAGGATCGTAAATTTGAA
     L  G  D  L  E  S  S  V  D  D  P  R  T  E  E  D  R  K  F  E
     A  .  .  .  .  .  .  .  .  .  .  S  .  .  .  K  R  .  .
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FIGURE 8. Nucleotide and predicted amino acid sequence of the PA21 capsid protein VP1. Numbering and cleavage sites are based on those of Cohen et al., 1987a. The differences present in the HM175 sequence are displayed above (nucleotides) or below (amino acids) the PA21 sequence.

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ABSTRACT (Report Number 3)

An infectious cDNA clone derived from virulent HAV would be of considerable
value in experiments designed to determine the molecular basis of attenuation,
and would provide the basis for new approaches to developing candidate
attenuated viruses. In an effort to assemble such a clone, partial inserts
from ten HM175 cDNA clones were assembled into a single construct containing
the consensus p16 HM175 sequence. Flanking homopolymeric dC-dG tails derived
from the original cloning procedure were removed, and the full length sequence
of p16 HAV cDNA was inserted between the HindIII and XbaI sites of the
transcription vector pGEM3. At the conclusion of the contract period, the
infectivity of this construct (pHAV/p16) was under evaluation. The sequence
of the P1 genomic regions of two plaque-purified, cytopathic variants of HM175
virus was determined from virion RNA; one of these variants was shown to be a
spontaneous neutralization escape mutant. A novel immunoaffinity-linked
nucleic acid amplification system (antigen-capture/polymerase chain reaction,
or AC/PCR) capable of the strain-specific detection of HAV in clinical
specimens was developed and evaluated. Molecular cloning and partial
sequencing of the genome of PA21 strain HAV was undertaken in an effort to
determine the extent of genetic divergence from human HAV.